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(71) Applicant: BIOCIRCUITS CORPORATION [US/US]; 1324 Chesapeake Terrace, Sunnyvale, CA 94089 (US).

(72) Inventors: RIBI, Hans, O.; 2455 Skyfarm Drive, Hillsborough, CA 94010 (US). LIU, Ben; 1515 Cormorant Court, Sunnyvale, CA 94087 (US). GAUB, Herman; Fakultät für Physik der TU München, Lehrstuhl für Biophysik E-22, D-85748 Garching (DE).

(74) Agents: ROWLAND, Bertram, L et al.; Flehr, Hohbach, Test, Albritton & Herbert, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).

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(54) Title: DIRECT AND INDIRECT MODULATION OF SPECTROPHOTOMETRIC CHANGES IN LIPID LAYER IN MEASURING ANALYTES

(57) Abstract

Methods are provided for the detection of an analyte involving binding of a binding ligand (analyte or analyte mimic) to a reciprocal binding member, where the chromatic shift has a result of binding of the binding ligand to the a polymerized layer is measured; or the effect of the binding of the binding ligand on the chromatic shift resulting from a change in the environment of the polymerized layer is measured. Changes in temperature, pH, or other mechanisms for triggering optical change in the polymer provide for chromatic shift. The binding of an analyte bound to a ligand which is bound to the polymerized layer is shown to modulate the chromatic shift resulting from the temperature change, pH change or other mechanisms capable of triggering an optical change in the polymer's chromatic properties.

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DIRECT AND INDIRECT MODULATION OF SPECTROPHOTOMETRIC CHANGES IN LIPID LAYER IN MEASURING ANALYTES

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Serial Number 08/084,884, filed June 29, 1993 a CIP of application Serial Number 07/627,027, filed December 13, 1990, now U.S. Patent No. 5,268,305, a CIP of 07/453,784, filed December 20, 1989, now abandoned, a CIP of 07/366,651, filed June 15, 1989, now Patent No. 5,156,810.

INTRODUCTION

Technical Field

The field of this invention is the detection of analytes using polymerized lipid layers.

Background

As the world has become more complex and as our understanding of different phenomena has increased, there has been a concomitant need to improve methods of measuring the wide variety of substances in use today. From the clinical laboratory, there has been increasing interest in being able to measure various substances in the doctor's office, the home, at bedside, in the field, as well as other sites. With the continuously increasing number of physiologically active substances, both naturally occurring and synthetic, there has been a desire to be able to measure the substances as indicative f the health status f an individual, for therapeutic dosage monitoring, for

research, and the like. The substances may be found in a wide variety f samples, ranging over numerous orders of magnitude in concentration for their dynamic ranges of activity, and further differ as to the ease with which one may detect their presence. An area which has only recently assumed substantial commercial importance and will be of increasing importance is the detection of specific nucleotide sequences. Nucleotide sequences find application in genetic counseling, forensic medicine, detection of diseases, and the like. There is, therefore, a wide diversity of opportunities to measure diverse substances from different sources with different sensitivities and for a wide range of purposes.

The methods for detection have ranged from radioactive labels, light absorption, fluorescence, chemiluminescence, agglutination, etc. Each of these methods has found application and has disadvantages as well as advantages over alternative methods. As yet, there has been no single method which has proven applicable in all situations. There is, therefore, substantial interest in devising new methods which may provide for significant opportunities in measuring compounds of interest, where the protocols, apparatus, or reagents may provide advantages over other techniques.

Relevant Literature

U.S. Patent No. 4,489,133 describes procedures and compositions involving orderly arrays of protein molecules bound to surfactant. J. Am. Chem. Soc. (1988) 110: 7571-7572 describes methods for forming multilayer thin polymerized films. Lieser *et al.*, (1979) 17: 1631-1644 describe the preparation, spreading behavior, multilayer formation, and polymerization phenomena of various long chain diacetylene monocarbonic acids. Bhattachargee *et al.*, J. Chem. Phys. (1980) 73: 1478-1480 report the effects of pH and electrolyte on the absorption and fluorescence spectra of polydiacetylenes. Chance *et al.*, J.Chem Phys. (1979) 71:206-211 report on the effects of increasing temperature on the optical properties of polydiacetylene films. Olmstead *et al.*, J.Phys. Chem. (1983) 87: 4790-4792 describe the blue to pink transition of a polydiacetylene film by raising the temperature of the film. Fouassier *et al.*, Israel J. of Chem. (1979) 18: 227-232 describe the photochemistry of the polymerization of multilayer polydiacetylenes. Kanetake *et al.*, J. Phys. Soc. of Japan (1985) 54: 4014-4026, Day *et al.*, Israel J. of Chem. (1979) 18:325-329, Mino *et al.*, Langmuir (1992)

8:594-598, and Mino *et al.*, Langmuir (1991) 7:2336-2341 describe col r transitions of polydiacetylene films.

Procedures for preparing polymerizable lipid layers as cast layers or liposomes may be found in Kuo and O'Brien, J. Am. Chem Soc., 1988 110, 7571; Kuo and O'Brien, J. Chem. Soc. Chem. Commun., 1990, 839-841; Kuo and O'Brien, Macromolecules, 1990, 23, 3225-3230; Kuo and O'Brien, Langmuir, 1991, 7, 584-589; and Rhodes et al., Langmuir, 1994, 10, 267-275.

Preziosi *et al.*, "Water Soluble Polydiacetylenes: Synthesis and Properties," Polymer Preprints, Amer. Chem. Soc. (1980) 166-168. Bhattacharjee *et al.*, "Visual Conformational Transitions of Water Soluble Polydiacetylenes: Effects of pH and Electrolyte on Absorption and Fluorescence Spectra," J. Chem. Phys. (1980) 73.

Atomic force microscopy is described in Marti *et al.*, "Atomic Force Microscopy of an Organic Monolayer," Science (1988) 293: 50-52.

SUMMARY OF THE INVENTION

Methods and compositions are provided for determining the presence of analytes using a polymerized layer or material capable of undergoing a spectrophotometric or optical change, *e.g.* chromatic shift, in relation to a change in its condition, wherein at least one element of such change will be specific binding of analyte or an analyte mimic ("binding ligand") to a specific binding member, which may be associated with the polymerized layer or which is part of a compound which can produce a change in the environment of the polymerized layer in relation to the binding event, resulting in a chromatic shift. The chromatic shift which is measured can result from: a change in the spectrophotometric properties of the polymerized layer caused by the binding of the binding ligand to the polymerized layer; the binding of the binding ligand to the compound which produces a change in the environment of the polymerized layer in relation to the binding of the binding ligand; or the effect of the binding of the binding ligand to the polymerized layer on the triggering of an enhanced or retarded optical change, as a result of a change in the environment of the polymerized layer or the reversal of such optical change during triggering. Various agents may be employed to

induce the triggering of the spectrophotometric shift. By comparing the results obtained with a sample to a control value, the presence of the analyte may be detected.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 provides data for four parameters used to study the effect of streptavidin binding on the color shift of an ethyl morpholin PDA film.

FIG. 2 provides data for four parameters used to study the effect of streptavidin binding on the color shift of a 5% biotin/1,2 propandiol pentacosadiynoic ester film.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods and compositions are provided for detecting the presence of analyte in a sample. The methodology uses detection of a chromatic or spectrophotometric shift in light either absorbed or fluoresced, in response to a binding event between the analyte or analyte mimic ("binding ligand") and a polymerized diacetylenic (dyine material or polymer) acid, where the spectrum (absorbance or fluorescence) of the polymerized layer changes as a result of: a change in the spectrophotometric properties of the polymerized layer caused by the binding of the binding ligand to the polymerized layer; the binding of the binding ligand to the compound which produces a change in the environment of the polymerized layer in relation to the binding of the binding ligand resulting in a change in the layer's ability to respond; or the effect of the binding of the binding ligand to the polymerized layer on the triggering of an enhanced or retarded optical change, as a result of a change in the environment of the polymerized lipid or the reversal of such optical change during triggering. By comparing the difference in the change between the presence and absence of sample, one can correlate the change with the presence of analyte.

The polymerized layer may be an insoluble material, e.g. later, or a soluble material or in a colloidal state in an aqueous solvent. As used in this application, the term lipid refers to monomers having a hydrocarbon chain of at least 6 carbon atoms in length. The lipids of the subject invention may comprise one or more diacetylene groups and either one, *e.g.* polar lipophilic monomers, or two polar head groups, where the monomers which comprise two polar head groups are proximal to the termini of the

linking aliphatic group, *e.g.* dual headed. However, either situation may be used for carrying out the analysis in accordance with this invention. The colloidal polymerized lipid, referred to as a lipid layer, may be prepared in a wide variety of ways, where the lipid composition may be homogenous or heterogeneous as to the nature of the lipids involved, the moieties bound to the lipids, and the like. Likewise, soluble polymer strands can be made using monomers containing two polar head groups. These compositions are referred to as soluble polymer lipids. For the lipid layers, the layers may be unilamellar or multilamellar films. In addition, one may form liposomes, where liposomes also fall within the definition of polymerized layer, having a conglomerated or aggregated structure, where the liposomes may then be coated onto an appropriate support, or remain in solution thereby avoiding the requirement for a solid support.

The manner in which the polymerized layers are prepared may be varied widely, although some procedures will be preferred over others. In addition, the composition of the lipids may be varied widely, where again some monomers may be preferred over others.

The polymerized films used in the method can be prepared from the above lipid monomers, for the most part, using conventional techniques and employing particular conditions to achieve the layers with desired qualities. Conventional Langmuir-Blodgett techniques may be employed. Soluble phase polymerization and subsequent solubilization can be employed. Methods for making vesicles, such as extrusion, can be employed. A large number of parameters are available which can be used to influence the nature of the product. These parameters include the buffer type, including pH, ionic strength, cations employed, *e.g.*, mono- or polyvalent, composition of the surfactant, both as to the polymerizable surfactant and the nonpolymerizable surfactant, including such considerations as chain length, the nature of the polymerizable functionality, the nature of the polar head group, the manner in which the surfactant layer is formed, including concentration of surfactant and solvent, the nature of the solvent, the spreading method, the amount of surfactant employed, subphase composition, superphase composition, all of which will affect the formation of mono- or multilamellar layers. Additionally, physical parameters, such as film tension,

crystallization time, temperature, humidity, traverse rates, will affect the nature of the polymerized film.

The monomers, which are to make up the polymerized layer used in the subject method, after being compacted in a layer, are then polymerized employing any convenient means, *e.g.*, ultra-violet light. Polymerization times are important for controlling the assay responsiveness of the layers, such as the films. Prolonged polymerization times (10 minutes) can lead to less responsive layers. Polymerization times between 20 seconds to 5 minutes can lead to more responsive layers dependent upon radiation intensity. Typically, times ranging from 1 second to 30 seconds give the greatest response. On the other hand, the degree of polymerization must be sufficient to provide a change in optical density which is readily determinable and allows for accurate measurement. The distance of the UV light source can also be a factor in formation of the polymerized layers. Distances typically range from 1 to 4 inches, usually 1-3 inches. The fluence of UV light on the surface will range from 1 mjoule/cm^2 to 100 mjoule/cm^2, more usually 30 mjoule/cm^2.

Other parameters, including the presence of inert gases or free radical initiators, can be used to control the polymerization rates and material responsiveness. Other initiation systems include combinations of light and light sensitive initiators, heat labile chemical initiators or the like. Such initiators are conventional and need not be described here. The activation is maintained until at least substantially complete polymerization is achieved. Polymerization may also be carried out by using electron beams, X-ray sources, synchotron radiation and the like.

Polymerization should occur in the absence of free oxygen. Accordingly, the film may be polymerized in the presence of inert gases, submerged in the subphase, or in any other environment where free oxygen is not present.

The polymerized layer or material can then be transferred to any convenient support for subsequent visualization of the change in absorbed or emitted light. The layer can be left in solution, placed on a fluid support, or transferred to a porous membrane or tape. When the layer is transferred to a less rigid support, greater flexibility may be achieved with regards to the amount of light that is absorbed or emitted as a result of an analyte binding event. Specifically, the polymerized groups experience a greater degree of flexibility than they do on a rigid support, thus allowing

for the possibility of reversible shifts in absorbed or emitted light. Transferring the layer to a highly rigid support may limit the film's flexibility, and thus reversibility, of the color shift. However, transferring the film to a porous surface can be used to draw the sample through the layer, thus concentrating the analyte on the film surface. This may enhance binding of the analyte to the ligand on the film surface.

Transfer of the layer to the various available supports, can be accomplished through any convenient means, particularly using Langmuir-Blodgett techniques [George L. Gaines Jr.: Insoluble Monolayers at Liquid Gas Interfaces, Interscience Publishers, I. Prigogine Editor, John Wiley and Sons (1964)]. The polymerized material can also be transferred to the support before polymerization, and polymerized while on the support. Following transfer of the polymerized material to the appropriate support, the polymerized material is ready to be used to detect the presence of analyte.

The various polymerized lipid compositions may be used, as appropriate, where one particular form may have advantages as compared to another. In selecting the particular form of polymerized lipid, considerations will include economies of manufacture, reproducibility, nature of the sample, responsiveness in the assay, stability to shipping etc.

The polymerized layer can be formed using the method described in U.S. application serial nos. 366,651, filed 06/15/89 and 453,784, filed 12/20/89, where a novel temperature gradient technique is employed. Surfactant films may be formed on the surface of an aqueous subphase by standard technologies for lipid monolayers, vapor deposited, cast, cast liposomal, spun, or gel phase. A solution containing a monomeric surfactant composition, dissolved in an organic solvent, is applied to the subphase surface by a micro-syringe. Solvents may include hydrocarbons such as pentane, hexane, heptane, and decane. The hydrocarbons may be straight chain, branched, cyclic, or unsaturated. Solvents may include chlorocarbons such as mono-, di-, tri- or tetrachloroethane. The addition of more polar solvents such as alcohols, furans, ethers, esters, or the like may be added to enhance the solubility of the surfactant composition.

The subphase composition is one process variable which dictates the physical characteristics of the surfactant layer which is formed. The subphase can be composed f pure water, glycerol, polyethylene glycol, or ther polar organic solvents miscible

with water including DMF, DMSO, acetone, alcohols, ketones, furans, dioxane, ethanolamine, phenols, colloidal substances including dispersed carbon powder, alone or in combination, or the like. High boiling point solvents such as glycerol will reduce evaporation during heating, while low boiling point solvents will enhance the evaporation. Other organic solvents can be used to stabilize the surfactant film, particularly to favorably interact with the polar headgroups, linkers and ligands of the surfactant. The subphase can also contain organic or inorganic acids or bases which affect the surfactant film through ionic interactions, *i.e.*, charge stabilization. The ionic components can include mono- and polyvalent ions and cations, and mono- and oligosaccharides.

Monomeric polymerizable surfactants are spread on the subphase at a concentration ranging from .01 to 50.0 mM in spreading solvent. Typically 0.1 to 5.0 mM is most useful. Films may either be formed from a homogenous solution of polymerizable surfactants or may be formed with a mixture of polymerizable surfactants and filler surfactants which have no polymerizable groups. The surfactants may or may not have ligands bound to the polar head group of the surfactant. The filler surfactant may have an hydroxyl, polyhydroxyl or polyethylene oxide headgroup which acts to prevent non-specific adherence of biological matter. When ligand is bound to the surfactant, the mole percentage incorporation of the ligand-surfactant to the filler-surfactant will vary depending on the particular assay, generally ranging from 0.01 to 100%, more usually from 0.1-10% and usually in the range of about 1.0 to 5%. Steric displacement can enhance protein binding, and steric hindrance can inhibit protein binding. The composition of the polar headgroup of the filler-lipid can thus provide a control mechanism for adjusting binding affinities and interactions, including responsiveness in an assay.

Monolayer film formation involves applying a subphase to a surface or well. A solution containing the monomeric surfactant is applied to a precleaned (aspirated) subphase surface until the surface is substantially saturated. The aqueous medium is pre-heated to melt and disperse the surfactant, usually to a temperature of not more than about 130°C, more usually not more than about 110°C, which results in evaporation of the spreading solvent. The medium is then allowed to cool to below room temperature, usually to about 7°C. The rate of cooling, a key process variable for

making highly crystalline films, is controlled by regulating the traverse rate of the subphase slide from the heating element to the cooling element. Typical traverse rates vary from .06 cm/min. to 1.0 cm/minute, usually 0.3 cm/min. Multilayer films can be formed by multiple film transfers to the same support surface or by modifying the film composition so that multiple layers can spontaneously form.

Cast films or liposomes may be prepared in accordance with the procedures described in the Kuo and O'Brien references described previously. The initial stage in providing the cast films or liposomes is to prepare an aqueous dispersion of the lipid, where the medium will comprise from about 0.1 to 5, more usually about 0.5 to 2 mM of lipid, buffer, *e.g.* Tris, PBS, HEPES, etc., and other additives, such as organic, polyvalent ions, glycerol, etc. The aqueous lipid dispersion is then sonicated in accordance with conventional ways, generally at a temperature in the range of about 20-60°C, for a time usually in excess of about 5 sec and not more than about 30 sec. Usually, a slightly opalescent suspension of vesicles results. To provide for individual vesicles, the water suspension may then be irradiated with UV light in the substantial absence of oxygen for sufficient time for the polymerization process to go to at least 50% completion, preferably at least about 75% completion. The polymerization process may be monitored spectrophotometrically. For uniformity of liposome size, the liposomes may be extruded, as described by Hope *et al.*, Biochimica Et. Biophysica Acta (1985) 812:55-65. For the most part, the liposomes will be of a size in the range of about 100 nm to several microns in diameter.

If a cast film is desired, the unpolymerized or polymerized vesicles may be spread onto an appropriate support, *e.g.* glass, nitrocellulose membranes, nylon membranes, paper or other porous materials, and allowed to dry, with substantially complete removal of water. If unpolymerized, the film may then be irradiated and the polymerization process followed spectrophotometrically, with the previously indicated degrees of polymerization desired.

For water soluble polymers, mono- or dioic diacetylenic compounds may be employed, particularly the dicarboxylic acids, where the carboxyl groups will be normally at the terminal positions. The carboxyl groups may be modified with a wide variety of hydrophilic groups, particularly uncharged or charged hydrophilic groups, such as oxy, amino, carboxy, inorganic acid groups, such as phosphorous acids, sulfur

acids, etc. and the like. Various polyols, polyethers, polyamines, polyacids, r combinations thereof may find use. The carboxyl groups may be unfunctionalized or functionalized, usually at least about 50 number % of the carboxyl groups being functionalized, depending upon the length of the chain, the nature of the binding group attached to the polymer, as well as the nature of the medium in which the assay is carried out. For soluble polymers, typically the diacetylenic monomers are crystallized from an organic solvent. The crystalline monomers are then polymerized in bulk using UV or X-ray irradiation. The polymerized material is then solubilized in an aqueous or organic media, the unpolymerized monomer extracted, and then the polymeric material processed for the assay. Sonication, detergents and other emulsifiers can be used to enhance solubility. Standard biochemical methods can be used to functionalize soluble polymers once they have been formed. Functional groups can then be used for attaching various ligands or binding members.

The surfactant or lipid molecule may have a single lipid chain, *e.g.* a diynoic acid or a plurality of lipid chains, e.g. diester glycerides, triester glycerides; mono- or polyesters of mono- or polycarboxylic acids, e.g. N-acyl bis (docosa-10, 12-diynil) L-glutamate; or cosa-8,10-diyn-1,20-dioic acid, and the like.

Various other surfactants may be present as diluents of the polymerizable surfactant. These surfactants may be naturally occurring, synthetic, or combinations thereof, and may be illustrated by laurate, stearate, arachidonate, cholesterol, bile acids, gangliosides, sphingomyelins, cerebrosides, or the like.

Various functional groups may be present in the material to provide for polymerization and various optical properties, such as Fõrster energy transfer. For the most part, the functional groups will comprise diynes, although other polyunsaturated molecules may find use, such as activated monoynes, *e.g.*, α-ketomonoynes.

For the most part, the hydrophobic portion of the polymerizable monomer will have a chain of at least 6 aliphatic carbon atoms, usually a straight chain of at least 8 carbon atoms, and generally not more than a total of about 100 carbon atoms, usually not more than about 34 carbon atoms. Preferably, the number of carbon atoms will vary from about 6 to 32, more usually 10 to 30, and more preferably 12 to 29 carbon atoms.

The monomers will terminate in a hydrophilic moiety, cationic, anionic r neutral (nonionic) where the functionalities may include non-oxo carbonyl, *e.g.*, carboxylic acids, esters and amides, oxo-carbonyl, such as aldehydes or ketones, oxy, such as ethers, polyethers, and hydroxyl, amino, such as primary, secondary, and tertiary amines and ammonium, phosphorus acid esters and amide, such as phosphate, phosphonate, and phosphonamide, sulfur functionalities, such as thiol, sulfonates, sulfate, and sulfonamides, and the like. Hydrophilic groups may include drugs, peptides, ligands, receptors, charge transfer complexes, or chromophores. Usually, the polymerizable functionality will be separated from the polar and non-polar termini by at least one carbon atom, generally from about 1 to 50 carbon atoms, more usually from about 1 to 8 carbon atoms. The polymerizable group may typically be incorporated into the hydrophobic interior of the surfactant film. The polymerizable group is typically a diacetylenic moiety, but other optical polymers may also be employed. The individual polymerizable groups can be spaced at regular intervals from 0-50 carbons apart, typically 0-10 carbon atoms apart, most usually joined by a bond. There can b as many of these groups in the chain as its length allows. Variations of the headgroup provide for variations in film properties, such as stability of the film, surface charge, control of interhead-group hydrogen bonding, reduction of non-specific binding or fluid matrix effects, and ease of chemical modifications. Single or multiple polymerizabl hydrophobic chains may be present per lipid unit. The polymerizable groups may also be incorporated between two polar head groups. These monomers may be polymerized in bulk crystals and then subsequently solubilized into soluble polymer strands. Their solubility is dictated by the polarity of the headgroups. The hydrocarbon tail of the surfactant may also terminate in a hydrophilic group so that the surfactant is bipolar. [Sher, Justus Liebigs Ann. Chem. (1954) 589:234; and Akimoto, et al. Angew. Chem. (1981) 20(1):91].

The ligand or binding molecule provides for specific binding pair member complex formation, where the amount of complex formation is related to the amount of analyte in the assay medium. The ligand or binding molecule may be associated with the lipid material, either directly or indirectly, being covalently bonded to the lipid material or covalently bonded to a macromolecule physically associated with the lipid material, r may be an entity whose activity is modulated as a result of complex

formation, e.g. an enzyme conjugate whose activity changes upon complex formation. Therefore, the ligand or binding molecule may take many forms, since the lipid material is a sensitive detector for a broad spectrum of events, such as direct binding and changes in its macroenvironment. The complex comprising the binding molecule, may involve the analyte, an analyte mimic or a molecule whose effective concentration varies with the amount of analyte, e.g. an antibody to the analyte.

The ligand or binding molecule used in analyte detection may be bound to an independent macromolecule or to the surfactant, where the preference will depend upon the nature of the polymerized material. With the material, the preference will be for an independent macromolecule, such as a protein, peptide, sugar, or other ligand, when referring to ligand, it is intended any molecule which specifically binds to another molecule. The macromolecule will be at least about 0.2kD, usually 50kD, or more and may be bound covalently or non-covalently to the support for the film or bound directly to the polymerized material. The macromolecule may be applied to a support before or after the polymerized material is applied to the support. The ligand can be chemically coupled, enzymatically coupled or absorbed to the polymerized material. The ligand density will range from .01 % to 100 % of the surface area. If the ligand is bound to the layer, depending upon the desired density of the ligand bound to the layer, the ligand size and the ligand's physical/chemical properties, the ligand may be present in from about 0.01 to 100 mol % of surfactant, more usually at least about 0.1 mol %, and preferably at least about 1 mol %, generally not more than about 10 mol %. The mol ratio will depend on the size and nature of the ligand, whether contiguous ligands are desired in the layer, and the like. The ligands may be joined by any convenient functionality, including esters, *e.g.*, carboxylate and phosphate, ethers, either oxy or thio, amino, including ammonium, hydrazines, polyethylene oxides, amides, such as carboxamide, sulfonamide or phosphoramide, carbons or polycarbons, combinations thereof, or the like. Specific groups may involve saccharides, both mono- and polysaccharide, including aminosaccharides, carboxysaccharides, reduced saccharides, peptides, polypeptides, nucleotides, oligonucleotides, or the like. Specific groups include zwitterions, *e.g.*, betaine, peptides, sugars, such as glucose, glucuronic acid, β-galactosamine, sialic acid, etc., phosphatidyl esters, such as phosphatidyl glycerol serine, inositol, etc.

The ligand or binding molecule can be any molecule, usually a small molecule, containing a reactive group. Typical ligands could be biotin, drugs such as alkaloids, chromophores, antigens, chelating compounds, crown ethers, molecular recognition complexes, polysaccharides, polypeptides, polynucleotides, ionic groups, polymerizable groups, fluorescence quenching groups, linker groups, electron donor or acceptor groups, hydrophobic groups or hydrophilic groups. The ligand may also serve as a site which can be further chemically modified to bring about new physical features or film characteristics.

The ligands or binding molecules which are covalently bonded to the surfactant will normally be a member of a specific binding pair. Thus, the ligands may be varied widely, usually being molecules of less than about 2 kD, more usually less than about 0.5 kD. For the most part, the ligands will be considered to be receptors or haptenic, which may include small organic molecules, including oligopeptides, oligonucleotides, saccharides or oligosaccharides, or the like. However, in some situations, the ligand bound to the surfactant may be a macromolecule, usually not exceeding about 500 kD, more usually not exceeding about 200 kD. Thus, proteins, nucleic acids, or other polymeric or nonpolymeric compounds of high molecular weight may also be employed. There is also the possibility to use crown ethers which will bind to particular ions. The particular manner in which one or more surfactants may be bound to the ligand is not critical to this invention and will depend, for the most part, on convenience, ease of synthesis, stability, available functional groups, and the like. Synthetic macrocyclic complexes may be incorporated into the surfactant layer for the purpose of molecular recognition of various natural and non-natural compounds.

In many cases, particular moieties will be used for a variety of purposes. For example, biotin may be used to bind to avidin or streptavidin, where the complementary member may then be used to link a wide variety of other molecules. Various lectins may be employed to bind a variety of sugars which may be attached to molecules f interest. Specific ligands may be employed which bind to complementary receptors, such as surface membrane receptors, soluble receptors, or the like.

Of particular interest is the binding of receptor, either directly or indirectly, to the surfactant. Direct binding will usually be covalent, while indirect binding will usually be non-covalent, such as non-specific or specific adsorption. Receptors of

particular interest will be antibodies, which include IgA, IgD, IgE, IgG and IgM, which may be monoclonal or polyclonal. The antibodies may be intact, their sulfhydryl bridges totally or partially cleaved, fragmented to $F(ab')_2$ or Fab, or the like. The intact and totally cleaved antibodies may be used to make a recombinant protein A-antibody hybrid, to be incorporated into the assay. Coupling through the antibody's oligosaccharide moiety to hydrazines can be achieved with the intact, partially and totally cleaved antibody. Maleimide linkages may be used for the intact, partially and totally cleaved antibodies, and the $F(ab')_2$ fragment, while the Fab fragment may be incorporated in an antibody hybrid. Other examples for antibody coupling to polymer films will include the use of recombinant hybrid linker proteins and recombinant antibody molecules. The antibodies may be functionalized at the Fc portion to ensure the availability of the binding sites for further binding. Other receptors include naturally occurring receptors, such as viral receptors, surface membrane protein receptors, blood protein receptors, etc.

In using the polymerized material for analyte detection, the analyte containing sample can be contacted to the polymerized layer using any convenient means. The analyte containing sample may or may not have been subject to prior treatment, such as removal of cells, filtration, dilution, concentration, detergent disruption to release antigen, centrifugation, or the like.

When the analyte in the sample should be maintained in an aqueous environment, the following procedure is found to be useful and may be treated as exemplary. An aqueous medium is formed, which is normally buffered at a pH in the range of about 4 to 9, preferably from about 5 to 9. The salt concentration will generally be in the range of about 10 mM to 1 M. Illustrative buffers include phosphate, borate, barbitron, carbonate, Tris, MOPS, MES, etc. Illustrative buffer compositions include phosphate buffered saline; 138 mM NaCl, 50 mM potassium phosphate, pH 7.2; 200 mM sodium borate, pH 8.2, etc. Use of polyvalent ions is often desirable. The concentration of the multivalent cations will depend to some degree upon the nature of the cation, generally ranging from about 0.1 to 200 mM, more usually from about 10 to 100 mM and will be included in the determination of total salt concentration.

The addition of detergents is often critical to reduce non-specific binding of the reagent to be coupled to the film particularly when the reagent is a protein. The amount of the detergent will depend on the nature of the protein, generally ranging from 0.001% to 10%, more usually from about 0.01% to 2%. Where non-specific adsorption of the binding member of the film is desirable, detergent may be left out. After submersing the polymer surface in an aqueous buffer containing from about 10-140 mM NaCl, 4-40 mM tris pH 6.5-7.5, as well as any additional appropriate coupling or other reagents and receptors, the reaction mixture is allowed to stand for sufficient time for completion of reaction, followed by washing.

The sample may be contacted to the polymerized layer by direct injection into a reservoir buffer covering the layer, by capillary action through a shallow flow cell covering the layer, by fluid pumping through a flow cell, by gas phase adsorption and diffusion onto a wetted surface covering the layer, or the like. For detecting extremely low concentrations of analyte, for example, less than about 10^{-12} M, the flow cell method or a porous membrane method is preferred, since it allows a large volume of sample to pass over the film surface so as to concentrate the specific binding member on the surface. At higher concentrations, a reservoir device configuration is useful, because the diffusion rate becomes less of a factor.

The binding event can be either direct or distal to the polymerized layer so long as there is associated with the binding event a change in the absorbed or emitted light of the layer. Thus, the change in chromatic signal can be as a result of the effect of binding of a specific binding member to the lipid material, the effect of specific binding of the specific binding member to the lipid material of a change in the chromatic signal due to an environmental change, or the effect of binding of a specific binding member to an entity, where the result of the binding is to produce an environmental change in the environment. The environmental change in the environment is normally a change in the macroenvironment of the lipid material, for example, the assay medium, irradiated light, or the like. In each situation, different pairs of specific binding members will be involved, where the formation of the specific binding pair complex results in the modulation of the characteristics of an entity, resulting in a chromatic shift different from the chromatic shift in the absence of the specific binding pair complex forming. The assays may involve binding of a binding

ligand (analyte or analyte mimic) to the lipid material or to a different material resulting in the modulation of the change in the environment of the lipid material with a resulting difference in the chromatic shift in the presence or absence of analyte. The binding ligand may provide for binding to the lipid material as the event providing the difference in the chromatic shift, inhibiting or providing for the binding of a different entity to the lipid material as the event providing the difference in the chromatic shift, or binding to an entity other than the lipid material to modulate the change in the environment of the lipid material as the event providing the difference in the chromatic shift, or the like. Therefore, the significant factor is that the subject system allows for a chromatic shift in absorption or fluorescence in relation to a binding event related to the amount of analyte in the assay medium, which binding event directly or indirectly results in a difference in the chromatic shift in the presence and absence of analyte.

A large number of coupling pairs may be employed, where the binding may be covalent or non-covalent. Various proteins which bind specifically to a complementary ligand may be employed, such as enzymes, lectins, toxins, soluble receptors, antibodies, and the like. Illustrative proteins include DHFR, streptavidin, avidin, cholera toxin, lectins, the c-H-ras oncogene product, enzymes, antibodies and nucleases. For linkages with oligosaccharides, hydrazine may be used, by itself or bound to a polymer, *e.g.*, poly(acrylhydrazide). Alternatively, biotin, nucleotides, or other molecular recognition analogs, or the like may be used. Nucleic acids, such as ssDNA or RNA may be employed. Maleimide linkages may be employed for linking to a thiol containing molecule, which may be biotin, avidin, any ligand or binding protein, sulfhydryl containing polymer, a nucleic acid, or molecular recognition analogs. For example, an intact antibody, with a functional oligosaccharide moiety, may be cleaved with periodic acid, and the resulting aldehyde reacted with the hydrazine under reductive conditions to form a stable carbon-nitrogen bond. For providing sulfhydryl groups to react with a maleimide, the antibody may be reduced at the hinge region, partially cleaved at the hinge region, or proteolytically cleaved near the hinge region for forming a thio ether with the activated olefin. In each case, care will be taken in selecting the method of linkage to ensure that the desired sites for binding to the complementary member of the specific binding pair are available for

binding. Alternatively, sulfhydryl surfactants may be attached to sulfhydryl groups n the antibody molecules.

When a molecule binds to a complementary ligand attached to the polymerized material the binding event can induce or modulate an absorbance or emission change in the material. In addition, various agents or processes can be used to enhance the optical change in the material due to the binding event. For example, the optical properties of polydiacetylene films or materials can be changed due to pH, temperature, mechanical stress (*e.g.* atomic force methods), various solvents, ionic strength, detergents, optical induction using a specific wavelength that the polymer may respond to, inorganic mediators or the like. The binding event may promote or retard the polymerized material's response due to pH, temperature, mechanical stress (*e.g.* atomic force methods), various solvents, ionic strength, detergents, optical induction using specific wavelength that the polymer may respond to, inorganic mediators or the like.

The binding event may also be distal to the polymerized layer and still be detectable by the layer. The binding event may occur in the ambient solution of the polymerized layer and result in a change in the layer's environment, *e.g.* a change in pH. By using hydrolases, e.g. phosphatases, glycosidases or esterases, products can be produced which result in a change in pH. The light absorbed or emitted by the polymerized film has been found to shift as a response to change in acidity of the ambient conditions of the film. For example, the light absorbed by a non-fluorescent blue form of polymerized film has been found to shift to a fluorescent red form as a response to a change in acidity. Using this finding, the binding ligand may compete for receptor with a binding ligand bound to an enzyme, where the enzyme conjugate comprises an enzyme which enzymatically converts a substrate to a composition which increases the pH of the medium. Such assay technique is described in U.S. Patent No. 3,817,837. Groups which may augment the pH include phenols, esters, carboxylates, and phosphates, but not nitrates or sulphates. This method may also be used for the detection of nucleotides, where the nucleotides have such groups attached to them and can serve as substrates. The binding event may also be distal and affect the shift in absorbed or emitted light of the polymerized lipid layer, where the analyte binds to the binding molecule which then binds to an antibody associated with the polymerized layer.

Instead of having the binding event provide for the major portion f the chromatic change, one may provide for an agent which induces a major change, which major change is modulated by the binding event. For example, changes in solvent, pH, ionic strength, mechanical stress (*e.g.* rubbing, atomic force microscopy or other mechanical perturbation) or other change in the environment of the polymerized lipid can provide for a substantial modification of the chromatic characteristics of the polymerized lipid. The effect of the change in environment can be modulated by the binding event, so that the binding event in conjunction with the change in environment can result in a greater response resulting from binding to the polymerized lipid. In this way, one can use a highly stable layer which is resistant to chromic change due to binding but which is specifically responsive to the environmental modification. In this case the binding event can be used to retard the optical responsiveness of the polymer due to the environmental modification.

With temperature, one may have a heating protocol or a combination of heating and cooling. In the case of the heating protocol, one may obtain a temperature profile over a range not exceeding 90°C, usually not exceeding about 60°C, preferably being from about 30 to 50°C. The rate of heating will generally be from about 0.5 to 50 °C per min, more usually from about 1 to 10°C per min. The reading may be anywhere from every 1°C up to about every 10°C, conveniently at 5°C intervals. One may measure various parameters associated with the temperature profile, such as mean temperature, steepness of transition, or other characteristics dependent upon the logic available for analyzing the data, the temperature range involved, the rate of heating, and the like. Alternatively, one may heat the solution, so as to provide for a substantial change from the original chromatic condition to the final chromatic condition, usually providing at least about a 60% change of the maximum change, preferably at least about 80% of the maximum change. One may then cool the polymerized lipid, using a similar schedule as described above and use the same characteristics for determining the effect of the analyte.

As already indicated, one may use pH, whereby the effect of the change in pH can be compared in the presence and absence of the analyte. Usually, the pH range will vary depending on the head group charge. A pH range of about 1 to 4 is used to trigger positively charged head groups and a pH range of about 8 to 11 is used to trigger

negatively charged head groups. Similarly, one may change ionic strength, where the ionic strength will vary from 1μm to 5 M, more usually from .1 mM to 50 mM. Ionic strength is dependent on the valence of the ions, *e.g.* monovalent or multivalent. With solvents, one may remove the aqueous solvent in which the binding occurred, optionally followed by washing and drying, and then followed by addition of a different solvent, particularly a non-acquiesced solvent, or one may add solvents to the original assay medium, where the solvents are dissolved in the assay medium or can displace the assay medium by contacting the polymerized lipid layer. Alternatively polar or non-polar solvents can be added directly to the aqueous media surrounding the polymerized material.

Mechanical methods such as rubbing or atomic force microscopy or other means of mechanical stress can be used to promote a conformational change in the polymerized material. The conformational change results in optical changes due to perturbing the polymer's orientation. Atomic force microscopy can be used in a tapping or scanning mode to selectively change regions of a polymerized layer from a blue, non-fluorescing form to a red, fluorescing form. The polymerized layer bound with protein will respond differently to mechanical force than an unbound polymerized layer. Thus, an assay utilizing a mechanical/optical triggering method can be employed.

The polymerized material can be exposed to specific wavelengths to which the polymer or other substituent can respond. Light/energy injected into the polymerized material results in a change in the material's conformational state due to interaction between the light/energy and absorbing conjugate polymer. Injecting light/energy can trigger the material to undergo an optical change from the blue form of the polymer to a red form of the polymer. This optical change can be used as an environmental change in the assay.

Since in many instances, the change in environment is found to provide a larger signal than the binding event, and the effect of the binding event on the change in signal resulting from the change of environment is greater than the change in signal as a result of the binding event, this triggering mechanism by changing the environment provides for a more sensitive assay. In other instances the binding event can inhibit the optical change in the polymer back bone due to the change in environment. In this case, a sensitive assay can be achieved by comparing a bound test film to an unbound control

film. After triggering through environmental change, the control film will change dramatically while the test film will be partially inhibited from change in relation to the amount of analyte bound to the polymer materials.

The method of detection focuses on the change in absorbed or emitted light of the polymerized layer which results from the presence of analyte. The spectral shift is the result of the change in absorbed light of the layer. For purposes of the method, it does not matter that the absorbed or emitted light is polarized or non-polarized. Alternatively, other spectroscopic methods can be used to measure the polymer's response to binding, *e.g.* IR spectroscopy, Raman spectroscopy, and other linear and non-linear techniques.

The color shift will normally be from blue to red. The red form is fluorescent while the blue form is not. Other color shifts can also be used in the method. Other possible color shifts, including red to yellow, are described in the referenced literature. Reverse chromism may also find use, *e.g.* yellow to blue.

It is found that different wave lengths can provide for more sensitive responses, in providing for greater changes in the chromatic signal. Particularly, it is found that greater sensitivity may be achieved by determining the change in ratio of the absorbance, for example, A636/A540 as compared to A646/A540.

The degree of absorbance shift in the polymerized layer may be proportional to the amount of analyte which is in the sample. The action of the analyte moiety binding to the film may cause a physical, chemical or electronic change in polymer/film composition. The binding event may induce or inhibit the film's ability to undergo a transition from one absorbance spectra to another. Likewise, the binding event may induce or inhibit the emission spectrum of the film. In both cases, the actual concentration of the binding events may be proportional to the degree of optical change that the film is allowed to undergo. The chromatic shift may result in a change in maximum absorption in the range of 565 to 850 nm to 400 to 560 nm.

The method may be varied so as to provide for signal enhancement. One form of signal enhancement focuses on the use of a polymerized multilayer film. The use of multilayers results in enhanced shifts making analyte detection easier. Alternatively, cascade enzymes which further enhance the signal may be used. Various mass or size dependent labels can be used to enhance the molecule's effect on the polymer.

Enzymatic processes can be used to amplify the signal change that the film can undergo. For example, a large analyte molecule may initially be bound to the film. In a sandwich assay configuration, a second antibody which contains an enzymatic label may bind to a second site on the analyte. Depending on the type of enzyme, the enzyme can be used to catalyze the formation of an abundance of product molecule which in turn reacts with the film and propagates a signal change in the absorbance or emission of the film. One enzyme product can be utilized by another enzyme already embedded or attached to the film providing for an amplification cascade. In certain polymerized layers, the binding event may elicit a cooperative response, where a small amount of analyte will trigger a shift in a large portion of the layer.

Different types of assays can be designed which fall within the scope of the method, so long as the presence of analyte is detected by a shift in the absorbed light of the polymerized layer. In the case of DNA assays, single-stranded DNA is immobilized in the film using one or two points of attachment. The attachment may be covalent or non-covalent, *e.g.*, biotin, avidin, hybridization, etc. When the sample containing a complementary strand of DNA is added, DNA duplexing leads to signal generation. In the case of a viral assay, virus capsid or envelope may bind directly to immobilized antibody or to specific viral receptors coupled to the film. Macromolecules will be assayed in a similar fashion to the viral assay. For serology, the same principle applies, but antigen is immobilized, and antibody is measured. Alpha-galactose-1,4-beta-galactose immobilized in the polymer film can bind to receptors of *P. fimbriea* bacteria.

Binding of the analyte may perturb the orderly packing leading to a structural change in the polymer layer. With polyvalent antigens cocking may be achieved, where cocking intends the binding of the polyvalent antigen results in bending of the film with a change in conformation of the polymer which results in the shift in absorbed light. Multivalent binding can be used to enhance the effect on the material. Similarly, large moieties, including gold particles, latex spheres, red blood cells, or the like can be used as labels to have a larger impact on the effective signal difference between bound and unbound analytes.

Two liposomes dispersed in the assay medium or used in forming a layer may have different binding m ieties such that a sandwich can be formed by bridging the

analyte between both liposomes. This has the added benefit when the liposome is dispersed in solution of increased specificity and potential for signal enhancement through agglutination.

The following experiments are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

The following experiments demonstrate shifts in emitted light of a polymerized film in response to both changes in ambient conditions and the binding of DNA.

Experiment 1. **Fabrication of a Multilayer Blue Film**

A glass microscope slide, 1 in. x 3 in. in area was neutralized in .22M KOH/Methanol solution for 2 hr. The slide was rinsed with Milli-Q water and dried with pure nitrogen. The slide was then made hydrophobic by treatment with dimethyl-n-octadecylchlorosilane. The slide was placed on a hot plate which was maintained at a temperature of 110°C. The hot plate was positioned next to a cold plate, maintained at a temperature of 7°C, which was separated from the hot plate by an insulating gap that was neither hot or cold. When the slide adjusted to the temperature of the hot plate, 1/6 of the slide was moved over the cold plate.

HPLC water was used as the liquid subphase. The subphase was placed on the warm side of the entire surface of the slide. The lipid monomer comprising a 2mM Ethyl Morpholin Pentacosadiynoic Amide/chloroform solution, was applied in a dropwise manner to the subphase with a micro-syringe. The chloroform acted as a spreading agent to ensure even spreading of the lipid monomer over the surface of the subphase. 100μl of the monomer were applied, which greatly exceeded the amount needed to form a monolayer over the surface and resulted in the formation of a multilayer. Because the excess concentration of monomer, multilayers were formed even though some monomer was displaced off of the slide.

The slide was then transferred from the hot plate to the cold plate at a rate of 0.3 cm/sec. Transferring the slide to the cold plate resulted in the formation of a highly uniform crystal orientation in the multilayer.The multilayer was then polymerized by irradiation with UV light (UVP, Inc. Model UVG-54 Mineralight) for 100 sec. The fluence of the light at the film surface was 30 mjoule/cm^2. The

distance of the film from the lamp was 2.5 in. Polymerization was carried out in an oxygen free environment, which ensured the formation of the blue multilayer film.

The resultant blue multilayer film was then transferred to a clean hydrophobic glass slide which had been made hydrophobic by treatment with dimethyl-n-octadecylcholorsilane, and maintained at room temperature for subsequent use in the detection of analyte.

Experiment 2. **Fabrication of a Monolayer Blue Film**

A glass trough (dimensions of 6 in. x 6 in. x 12 in.) was filled with 1.0 L of 0.0°C Milli-Q water. As above, a 2.0 mM ethyl morpholin pentacosadiynoic amide monomer(EMPDA)/chloroform solution was applied to the surface of the water in a dropwise fashion by use of a micro-syringe. Care was taken to add just enough lipid monomer to form a single lens over the surface of the water. In order to form a single lens, 25 μl were added.

A (1 in. x 3 in.) glass microscope slide was made hydrophobic by treatment with dimethyl-n-octadecylchlorosilane. The upper surface was then placed face down onto the surfactant lipid monolayer and pushed below the surface of the water. The hydrophobic portion of the surfactant lipid monomers previously on the surface adhered to the slide. Excess lipid monomer which did not adhere to the slide was aspirated off of the water surface.

The slide was then rotated by 180° under the water so that the hydrophilic groups were facing upwards. The monolayer was kept submerged in the Milli-Q water at a distance of .5 inches from the surface of the water.

Polymerization was by irradiation with a 250 UV lamp (UVP Model UVG-54 Mineralight) for 100 sec. at a distance of 1.5 in. from the surface of the water (2.0 in. from the monolayer surface). The fluence of light at the subphase surface was at 30 mjoule/cm^2.

Experiment 3. **Blue to Red Color Shift of Films in Response to Change in Ambient pH**

The blue films described above were observed to undergo a color shift from blue to red in response to changes in the ambient pH of the films. The color shift was demonstrated as follows. First, a 1 in. x 3 in. slide with a blue film, as prepared above, was placed on a white background. Following, several solutions with pH

values of .8, 1.2, 2.2, and 2.8 were made using 1M H_2SO_4 and the appropriate amount of Milli-Q water. The solutions of various pH values were pipetted onto the blue film and the following data was obtained.

pH	Film Color	Fluorescence (at 540 nm excitation)
0.8	Blue	None
1.2	Blue	None
2.2	Blue	None
2.8	Red	Emission at : 565 - 650 nm

As demonstrated in the above table, decreasing the acidity of the ambient pH resulted in a shift in the absorbed or emitted light of the polymerized layer, so that the layer shifted colors from blue to pink and became fluorescent during the transition.

Experiment 4. **Color Shift in Response to Binding of DNA to Ethyl Morpholin PDA Film**

An assay for the presence of DNA in a sample was run as follows.

A. Formation of the Flow Cell

A flow cell used to detect the presence of DNA in a sample was manufactured by first placing an 8-well flow cell made of double sticky silicon rubber spacer on the surface of a 2 in. x 3 in. microscope slide. The microscope slide served as the bottom of the flow cell. Following, a 1 in. x 3 in. glass slide which has the blue polymerized film, as produced above, was placed on top of the rubber spacer. Each well in the finished flow cell had a volume of 30 μl. Since the top slide was smaller than the bottom slide, a portion of each cell was exposed so that sample could be added to each well.

B. Detection of the Oligonucleotide

The flow cell was placed into an LED fluorescence monitoring unit and the background fluorescence of the cell was measured. Following, 4 of the wells were filled with 30μl samples of 2.6 x 10^{-4} M oligonucleotide/sterile water (21-mer: 5'-

LGG CAG-TTA-TCT-GGA-AGA-TCA-3'). The remaining 4 wells were filled with sterile water to serve as controls.

The flow cell was incubated for 20 min. at 53°C to allow for binding of the oligonucleotides to the film to occur. Following incubation, the solution was removed from each well and the flow cell was dried using highly purified nitrogen gas.

The flow cell was then placed back into the EPI fluorescence monitoring device and the fluorescence was measured. The fluorescence was found to increase in the wells which had DNA oligonucleotides present. Since the blue film does not fluoresce but the red film does, it was concluded that a shift in the absorbed light must have occurred in response to the binding of the DNA to the film. Control wells containing no DNA showed differentially 50% lower response compared to wells containing the DNA.

Experiment 5. **Color Shift in Response to Binding Streptavidin to Ethyl Morpholin PDA Film in Relation to Varying UV Polymerization Time.**

A. Preparation of Film.

A 5% biotin attached to 3 repeat Polyethylene glycol spacer/95 % ethyl morpholin PDA film was prepared by spreading the lipid mixture at the air-water interface with stirring at a sub-phase temperature of 10°C and compressed to 25 mNt/m. The film was manually and horizontally transferred to 4/1x3" slides stuck to a holder. After being air-dried, the films were polymerized by placing the holder about three inches beneath a xenon arc lamp and turning on the lamp for 1, 5, 15, or 45 sec. The slides were stored in a box at room temperature.

The slides were assembled in 8-well devices using double-sticky tape on glass 2x3's. Each well was scanned from 350-800 nm ("pre-binding"). The wells for each slide were then filled as follows: wells 1 and 8 with deionized ("DI") water, wells 3, 5 and 7 with TBS (Tris buffered saline), and wells 2, 4 and 6 with Streptavidin ("SA") at 15 ug/nl in TBS. The slides were incubated for 20 min and then rinsed 3x with TBS and 3x with DI water. The slides were then scanned

("post-binding") to determine the extent that SA modified the chromatic properties of the film.

B. Determination of the Effect of Streptavidin Binding on Temperature Triggering.

Temperature triggering was done by filling each well with DI water and submerging each slide in a trough containing DI water, which was itself placed in a circulating water bath at a fixed temperature for one min. The slides were then removed from the bath, blown dry with air (at this point they were at room temperature) and then scanned. The process was then repeated at higher temperatures in the range from 20 to 80°C at 5°C increments.

The data was analyzed by first determining that the absorbance peaks for the blue and red forms of the film occurred at 646 and 540 nm, respectively. Each scan was corrected for baseline variation by subtracting the flat portion of the scan around 750 nm. The ratio A646/A540 (R) was then computed and plotted versus temperature. The curve was fitted with a 4-parameter sigmoidal curve model using the program Tablecurve.

All three solutions (water, TBS and SA) trigger the film directly to a small extent. Statistically, there were no significant differences between the three treatments at any of the UV times, however for all 16 slides, the mean of the ratio post- and pre-binding was lower for SA than for TBS.

Considerable overlap in the curves for water, TBS and SA were observed, with the exception that for one second UV polymerization, SA appeared to inhibit the transition.

Data was normalized to the 20°C scan, to provide an initial value of 1, in order to correct for differences due to the film's initial state or its state following direct triggering. At one second and five second UV, SA has an inhibiting effect, but there appears to be no obvious difference from water or TBS at the other two time periods. The between-slide variation in R decreases at lower R values, where R is defined as the ratio of maximum absorbance for the blue form to the maximum absorbance f r the red form. This implies that although the slides may start out with

varying degrees of "blueness" (as measured by A646/A540), they end up with the same degree of "redness". Normalizing the data improved the goodness of fit, as measured by either r^2 or the F-statistic; h wever, it did not significantly change the fitted parameter values.

The fitted parameter values are shown in FIG. 1.

Parameter a corresponds to the final R value, *i.e.* when the film has completely triggered ("final redness"). Parameter b corresponds to the initial R value. Parameter c corresponds to the inflection point halfway between the initial and final states. Parameter d corresponds to the steepness of the curve, *i.e.* the temperature range at which R goes from to 37% to 63% of its final value; the smaller d is, the more uniform the triggering.

There were no significant differences in a between SA and TBS. b appeared to be lower for SA than TBS, which is consistent with SA causing direct triggering *i.e.*, the SA-treated films started out "redder" than the TBS films prior to elevating the temperature. c was consistently higher for SA, indicating that SA inhibits the transition. This effect was seen at all four UV times, although the difference was greatest at one sec UV and decreased with increasing UV time. d didn't appear to be significantly different for SA than TBS, but increasing UV time resulted in smaller values for d or more uniform triggering.

Experiment 6. Determination of Streptavidin Using 5%Biotin/1,2-Propandiol Pentacosadiynoic Acid Ester Film.

5% biotin/diol ester PDA films were prepared by spreading the lipid mixture at an air-water interface with stirring with nitrogen at a subphase temperature 40°C and compressed to 25mNt/m at 0.01cm/s. The film was manually and horizontally transferred as described in the previous experiment, where the glass was silanized. The procedure was repeated to form a bilayer. The transfer was followed by air drying and polymerizing by placing the holder of the slides about three inches beneath a xenon arc lamp and exposing the film for 1, 5, 15, or 45 sec. The slides were then stored at room temperature.

The slides were assembled in 8-well devices as described above, except that the slides were air blown dry after the washings. Each well was scanned from 350-800 nm ("pre-binding") and then filled in the same manner as described above. After incubating for 20 min, and rinsing 3X with TBS and 3X with DI water, the slides were then scanned again ("post-binding"). The effect of SA was determined by this procedure.

Temperature triggering was done by filling each well with DI water and submerging each slide in a trough containing DI water, which was itself placed in a circulating-water bath at a fixed temperature for one min. This lodge was then removed from the bath, blown dry with air and then scanned. The process was repeated in the range from 20 to 80°C at 5°C increments.

The results were analyzed as previously described, except that the red peak employed was 536 nm. Each scan was corrected for base line variation by subtracting the flat portion of the scan around 750 nm. The "direct effect" was measured by the R post-scan divided by R pre-scan for each well. The three different solutions for each slide were averaged and then the four slides at each UV time were averaged. For all UV times, SA wells had a lower post-binding/pre-binding ratio compared to TBS or water, the difference was only statistically significant at 1" and 5" UV. At 1" UV, SA treatment led to a 70% decrease in R vs. only 35% for either TBS or water. At 5" UV, SA treatment resulted in a 50% decrease in R s. 30% for TBS or water. The "indirect effect" or triggering was looked at by plotting R against temperature and using Tablecurve to fit a four parameter sigmoidal curve as described previously.

FIG. 2 provides the results for the four parameter values.

Theoretically a should not go below zero, but for SA and TBS at one " UV it did. For b, the biggest difference is seen in the 1" UV time. The results with b indicate that increasing UV time leads to films with lower initial values; however, regardless of the initial R value, the "direct" effect of SA binding is to convert all the films to approximately the same final R value. The results with c showed that in

all cases SA binding inhibited the temperature-triggered transition relative to TBS, with the biggest difference for 1" UV.

The conclusions are that there is a greater "direct effect" or conversion from blue to red film, due to SA binding as compared to TBS or DI water. The difference was statistically significant at 1 and 5" UV times. The results with the subject polymerized layer were analogous to the layer of Experiment 5, but more pronounced. SA inhibited the chromatic shift more than TBS or DI water, with the greatest difference at 1" UV.

It is evident from the above discussion and experiments that a simple, easy to use methods for the detection of a wide variety of analytes are provided. The methods focus on the shift in the absorbed or emitted light of a polymerized layer. The shift is a direct result of the binding of an analyte to the layer. By employing a wide variety of ligands in the layer, a wide variety of analytes can be detected using the above method. The method is not limited to the detection of a visual color shift of the layer, but can also use the appearance of fluorescence on the layer.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for detecting in an assay medium the presence of an analyte in a sample using a polymerized material and a binding molecule associated with said polymerized material or dispersed in said assay medium, wherein said binding molecule is a member of a specific binding pair capable of binding to said analyte and said polymerized material undergoes an optical change as a result of specific binding to said binding molecule and upon a change in the environment of said polymerized material, said method comprising:

contacting said polymerized material with an assay medium comprising said sample suspected of containing said analyte for sufficient time for specific binding by said binding ligand to said specific binding member;

optionally changing the environment of said polymerized material; and

determining the effect of said binding of said binding ligand to said specific binding member on said optical change as an indication of the presence of said analyte in said sample.

2. A method according to Claim 1, wherein said binding moleucle is associated with said polymerized material and the effect of specific binding to said specific binding member on said optical change is determined.

3. A method according to Claim 1, wherein said binding moleucle is associated with said polymerized material and the effect of binding to said specific binding member on the optical change as a result of a change in environment is determined.

4. A method according to Claim 3, wherein said change in environment is a change in temperature.

5. A method according to Claim 3, wherein said change in environment is a change in pH.

6. A method according to Claim 3, wherein said change in environment is a change in ionic strength.

7. A method according to Claim 3, wherein said change in environment is a change in solvent.

8. A method according to Claim 3, wherein said change in environment is due to mechanical stress of the polymerized material.

9. A method according to Claim 3, wherein said change in environment is due to exposure of said polymerized material to light of a specific wavelength.

10. A method according to Claim 1, wherein said polymerized material is produced by polymerizing a layer of dyine monomers on an aqueous subphase.

11. A method according to Claim 1, wherein said polymerized material is produced by spreading aggregated diyne monomers on a solid support to form a substantially uniform precursor layer and polymerizing said precursor layer.

12. A method according to Claim 1, wherein said polymerized layer comprises liposomes.

13. A method according to Claim 1, wherein said polymerized layer is a soluble polymerized layer.

14. A method according to Claim 1, wherein said optical change is a chromatic shift, wherein said chromatic shift results in a change in maximum absorption in the range of 565 to 850 nm to 400 to 560 nm.

15. A method for detecting the presence of an analyte in a sample using a partially polymerized lipid layer and a binding molecule associated with said polymerized lipid layer, wherein said binding molecule is a member of a specific pair capable of binding to said analyte and said polymerized lipid layer undergoes a chromatic shift upon specific binding to said binding molecule, said method comprising:

contacting said polymerized lipid layer with a sample suspected of containing said analyte for sufficient time for specific binding to said specific binding member; and

determining the effect of said binding to said binding molecule on said chromatic shift as an indication of the presence of said analyte in said sample.

16. A method according to Claim 15, wherein said binding molecule is covalently attached to said polymerized lipid layer.

17. A method according to Claim 15, wherein said binding molecule is covalently attached to a macromolecule associated with said polymerized lipid layer.

18. A method for detecting the presence of an analyte in a sample using a partially polymerized lipid layer and a binding molecule associated with said polymerized lipid layer, wherein said binding molecule is a member of a specific pair capable of binding to said analyte and said polymerized lipid layer undergoes a chromatic shift upon specific binding to said binding molecule or a change in the environment of said polymerized lipid layer, said method comprising:

contacting said polymerized lipid layer with a sample suspected of containing said analyte for sufficient time for specific binding to said specific binding member;

changing the environment of said polymerized lipid layer; and

determining the effect of said binding to said binding molecule on said chromatic shift as an indication of the presence of said analyte in said sample.

19. A method according to Claim 18, wherein said change in environment is a change in temperature.

20. A method according to Claim 19, wherein said change in temperature is a change of at least 30°C.

21. A method according to Claim 18, wherein said polymerized lipid layer is in contact with said sample during said change in environment .

22. A method for detecting in an assay medium the presence of an analyte in a sample using a partially polymerized lipid layer and a binding molecule associated with an entity dispersed in said assay medium, wherein said binding molecule is a member of a specific pair capable of forming a complex resulting in a change in the activity of said binding molecule, wherein said binding molecule is capable of changing the environment of said polymerized lipid layer producing a chromatic shift upon specific binding to said binding molecule, said method comprising:

contacting said polymerized lipid layer with said assay medium comprising said sample suspected of containing said analyte for sufficient time for specific binding to said binding molecule resulting in a change in the environment of said polymerized lipid layer; and

determining the effect of said binding to said binding molecule on said chromatic shift as an indication of the presence of said analyte in said sample.

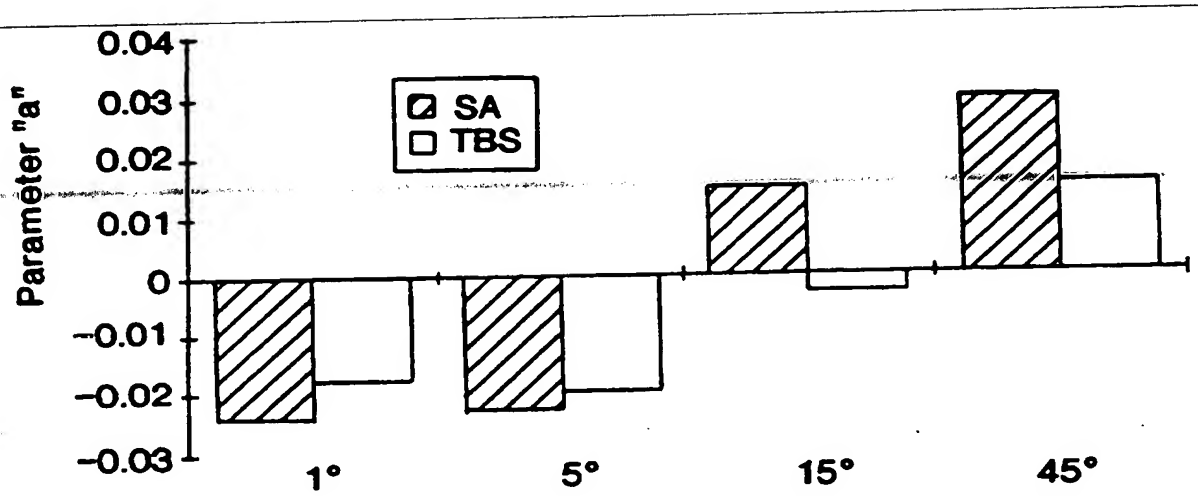

FIG. 1A

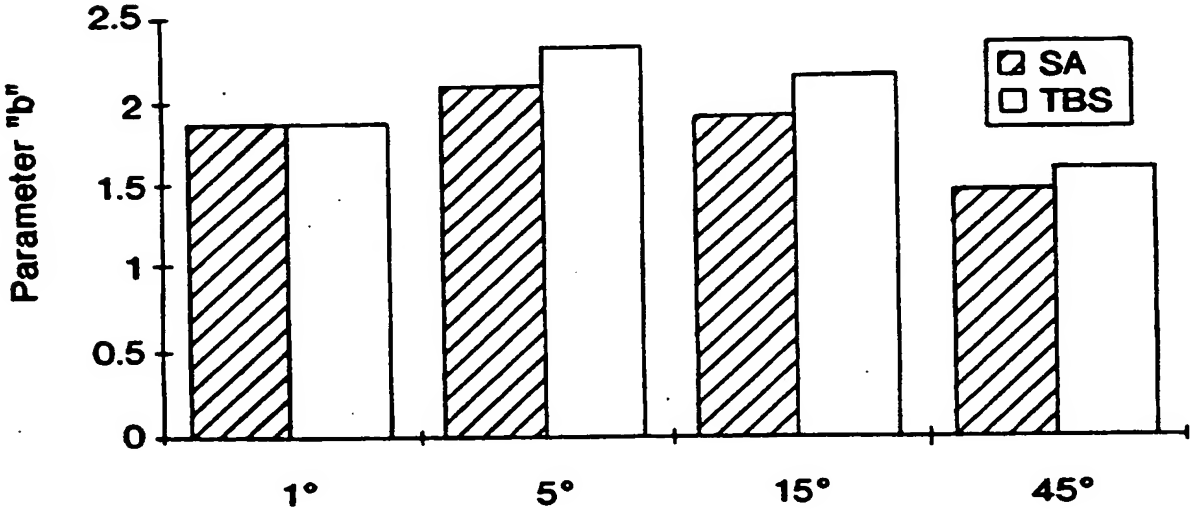

FIG. 1B

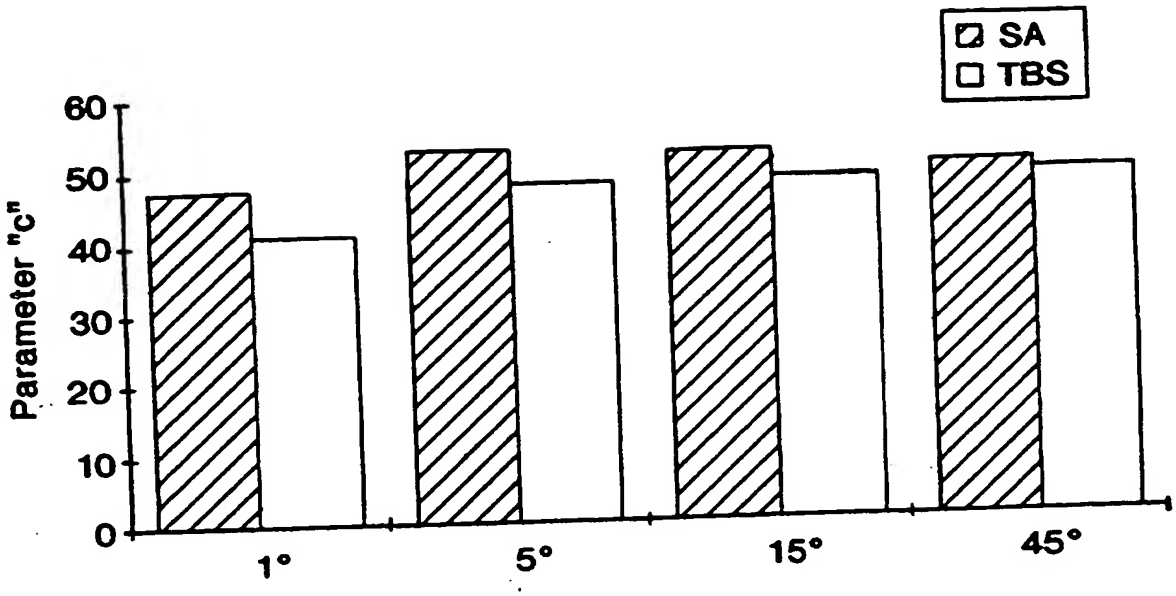

FIG. 1C

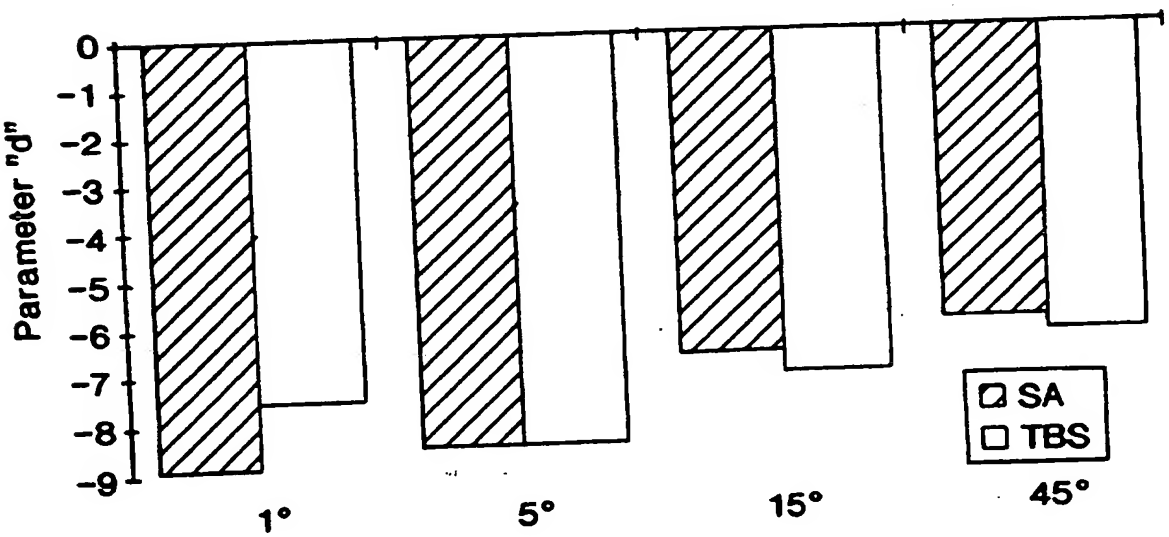

FIG. 1D

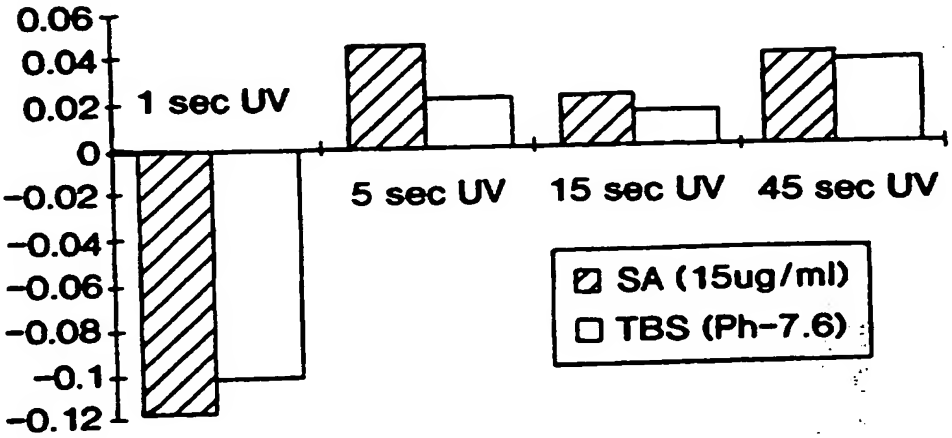

FIG. 2A

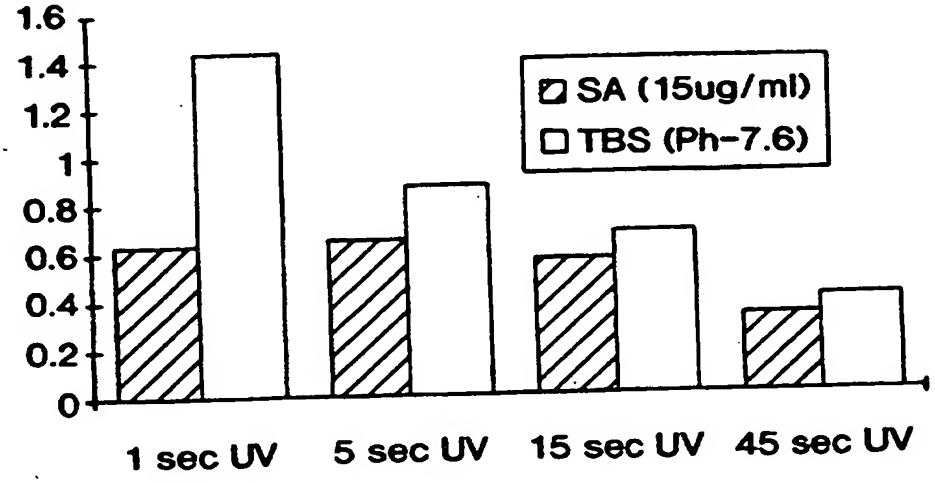

FIG. 2B

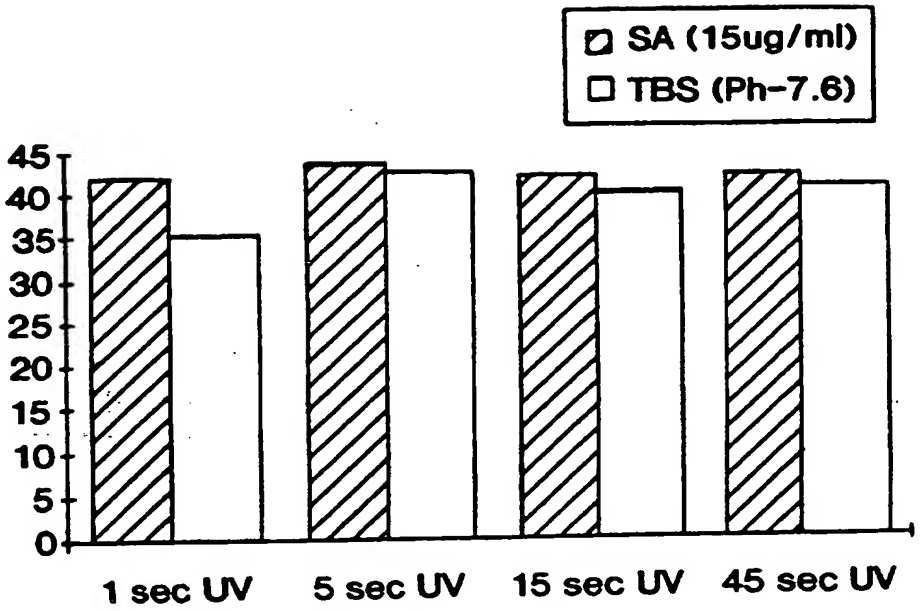

FIG. 2C

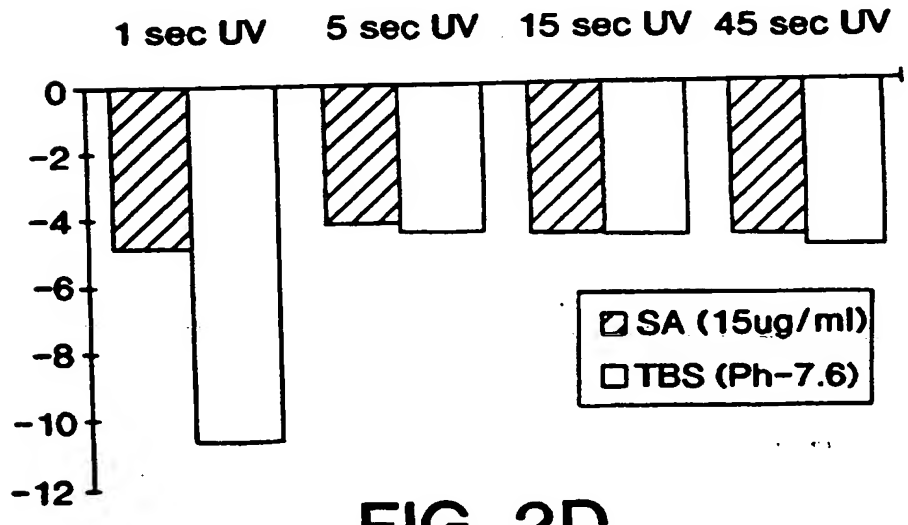

FIG. 2D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11672

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/543
US CL :436/518
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/287,289,291;
436/164,169,172,518,528,531,532,805

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS
search terms: polymerized lipid layer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4,489,133 (KORNBERG) 18 December 1984, see entire document.	1-3,9, 15-18, 21-22
X,P	US, A, 5,415,999 (SAUL ET AL) 16 May 1995, see entire document.	1-3,13, 15-18, 21-22
X,P	US, A, 5,399,486 (CATHEY ET AL) 21 March 1995, see entire document.	1-3,13, 15-18, 21-22
X,P	US, A, 5,427,915 (RIBI ET AL) 27 June 1995, see entire document.	1-3,13, 15-18, 21-22
X	US, A, 5,268,305 (RIBI ET AL) 07 December 1993, see entire document.	1-3,13, 15-18, 21-22
X	US, A, 5,156,810 (RIBI) 20 October 1992, see entire document.	1-3,10,11, 13,15-18, 21-

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
06 DECEMBER 1995

Date of mailing of the international search report
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Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile N . (703) 305-3230

Authorized officer
CHRISTOPHER CHIN

Telephone No. (703) 308-0196

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